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INCLUSIVE DATES___1 February, 1962___TO___31 January, 1963_____

# SUBJECT OF INVESTIGATION

THE BIOLOGICAL SIGNIFICANCE AND CHEMISTRY

OF

A PROTEASE INHIBITOR NEWLY ISOLATED

FROM

ANIMAL TISSUES

## RESPONSIBLE INVESTIGATOR

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In previous studies we have found that the euglobulin fraction, prepared from the subsiding or healing site of cutaneous Arthus inflammation in rabbits, can suppress markedly the inflammation; and the suppressing effect of the protein fraction is largely due to a specific protease inhibitor present in the protein fraction.

This previously undescribed antiprotease was extensively purified and obtained as fibrous crystalline-like substance. This antiprotease inactivated a particular SH-dependent protease of the cutaneous lesion of Arthus inflammation

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and papain, but had no effect on trypsin and chymotrypsin. It appeared to be a polypeptide, positive with biuret test, thermostable, nondiffusible, non-precipitable with 5% trichloroacetic acid and insoluble in water but soluble in salt.

The mechanism of increased vascular permeability in inflammation was studied because this phenomenon is one of the cardinal features in inflammation. Increased vascular response in cutaneous Arthus inflammation was diphasic—immediate and delayed; the former was temporary and mediated by a substance susceptible to antihistamine (possibly histamine); the latter was intense and prolonged, and mediated by other systems.

A new permeability factor (PF) was isolated as the pseudoglobulin fraction from the lesion showing the delayed response; the substance was susceptible to antihistamine, soy bean trypsin inhibitor, or di-iso-propyl phosphofluoridate, and had no smooth muscle-stimulating action of chemotactic action on leucocytes. (Author)

# D-I-S-T-R-I-B-U-T-I-O-N

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# A B S T R A C T

## 1. Purification of a protease inhibitor:

In previous studies we have found that the euglobulin fraction, prepared from the subsiding or healing site of cutaneous Arthus inflammation in rabbits, can suppress markedly the inflammation; and the suppressing effect of the protein fraction is largely due to a specific protease inhibitor present in the protein fraction.

This previously undescribed antiprotease was extensively purified and obtained as fibrous crystalline-like substance; the purification procedures essentially consisted of fractionation with ammonium sulfate, chromatography with Sephadex G-50 and DEAE-cellulose, and concentration with Sephadex G-25. This antiprotease inactivated a particular SH-dependent protease of the cutaneous lesion of Arthus inflammation and papain, but had no effect on trypsin and chymotrypsin. This substance appeared to be a polypeptide, positive with biuret test, thermostable, non-diffusible, non-precipitable with 3 % trichloroacetic acid and insoluble in water but soluble in salt.

## 2. Endogeneous mechanism in vascular permeability in inflammation:

The mechanism of increased vascular permeability in inflammation was studied because this phenomenon is one of the cardinal features in inflammation. Increased vascular response in cutaneous Arthus inflammation was diphasic--immediate and delayed; the former was temporary and mediated by a substance susceptible to antihistamine (possibly histamine*); the latter was intense and prolonged, and mediated by other systems.

A new permeability factor (PF) was isolated as the pseudoglobulin
fraction from the lesion showing the delayed response; the substance
was insusceptible to antihistamine, soy bean trypsin inhibitor, or
di-iso-propyl phosphofluoridate, and had no smooth muscle-stimulating
action or chemotactic action on leucocytes. The permeability-increasing
effect of the pseudoglobulin was suppressed markedly by the euglobulin
prepared following the same way as noted above. Inspite of the exis-
tence of the antiprotease, the suppressing effect of the euglobulin
was possibly due to a new substance (anti-PF). Partial purification
of this anti-PF was made by using Sephadex G-50 column.

Those observations presented here are very suggestive for clarifying
the important endogeneous mechanisms of various tissue manifestations
in inflammation.

APPENDIX "A"

THE BIOLOGICAL SIGNIFICANCE AND CHEMISTRY OF A PROTEASE INHIBITOR
NEWLY ISOLATED FROM ANIMAL TISSUE

1. PURIFICATION OF A PROTEASE INHIBITOR

The possible role of proteases in the allergic response, and in tissue damage in general, has received increasing attention, particularly in the last decade (see review by Ungar and Hayashi, 1958).

Oonishi (1942) observed increased proteolysis in the lungs of dogs in which allergic pneumonia was induced; and Herberts (1955) detected a protease, inactivated by sulphydryl block, in the lungs of guinea-pigs in anaphylactic shock. Increased proteolysis also occurs in other organs; e.g., in the liver of frogs in peptone shock (Weiner, 1955).

Previous studies in this laboratory have shown that the activity of certain skin protease increases in parallel with the development of cutaneous Arthus-type reactions; and that in activation of the protease by a polypeptide inhibitor may explain the subsequent and gradual cessation of the reactions (Hayashi, 1955, 1956, 1958; Hayashi et al., 1958; Udaka, 1960; Ito, 1960). The protease and anti-protease each were released by tissue phagocytes during antigen-antibody reactions, the enzyme being released befor its inhibitor. Both substances were recovered in euglobulin fractions (Hayashi, Tokuda and Udaka, 1960; Tokuda, Hayashi and Matsuba 1960; Udaka, 1960).

The reactions were suppressed by a crude preparation of euglobulin containing the inhibitor (Matsuba, 1960); or by more refined preparation (Miyoshi, unpublished) injected with the antigen. These preparations also were effective when given either before or after injecting the antigen. Accordingly, the protease may be one of the factors responsible for the inflammatory lesion, the balance between the protease and its inhibitor det mining the intensity, extent and duration of the reactions. The inhibitor can inactivate papain but has no effect on trypsin or plasmin (Udaka, Harada and Hayashi, 1961).

This paper describes further observations on purification of the inhibitor by a column chromatographic technique and on some properties of this inhibitor.

## EXPERIMENTALS AND RESULTS

Adult male albino rabbits (2.0 - 2.3 kg.) bred in our laboratory were used.

**Sensitisation of animals.** --- Five ml. of bovine serum was injected subcutaneously on the 1st, 3rd and 5th days. On the 16th day the antibody content of each animal's serum was determined by precipitin test (Hayashi et al., 1960). In tubes 3 mm. diam., serial dilutions of serum were tested with equal volumes (0.1 ml.) of antigen, and results read after 6 hr at room temperature. Only animals with titres of $2^6$ were used.

**Induction and evaluation of Arthus-type inflammation.** --- The backs of sensitized animals were closely clipped. After 24 hr. 0.2 ml. bovine serum was injected intradermally in 20 sites in an area 15 x 12 cm. The serum induced Arthus-type reactions which were equally intense at all sites.

Macroscopically, the reactions commenced in about 3 hr., were maximal in 22 - 26 hr., and then declined in intensity. The inflammatory reaction had usually disappeared 12 - 19 days after injection. Animals with such reactions were used for the test but were discarded if healing of the lesions was delayed at any stage by sloughing of the skin.

**Preparation of skin acetone powder.** --- Healing inflamed skin was excised immediately after killing the animals. Fat and muscle was removed, the skin finely minced with scissors and immediately frozen at $-80^\circ$. The frozen skin was cut into slices about 50 $\mu$ thick with a freezing microtome and the slices placed in 2-4 l. of cold acetone of which the temperature then was allowed to rise to $2^\circ$. Dehydration was continued with 3 changes of acetone (2-4 l. ), each batch of acetone being held with the skin for 60 min. at $2^\circ$, and then removed by filtration. The powdered skin was then air-dried in a hood to remove residual acetone and desiccated in vacuo over $P_2 O_5$ for 24 hr. The technique was described by Udaka (1960).

**Preparation and use of Sephadex and DEAE-Cellulose:**

Sephadex G-50 and G-25, employed in this study, were purchased from commercial souces (Pharmacia, Uppsala, Sweden) and prepared by the method of Porath and Flodin (1959), and Flodin, Gelotte and Porath (1960). DEAE-cellulose, containing 0.87 meq. of titrable groups per g., was purchased from commercial souces (Carl Schleicher & Schnell Co. Ltd.) and prepared according to Peterson and Sober (1956).

The columns were packed as follows;

Column I. —— Air-dried Sephadex (G-50, about 10 g.) was suspended in 25 volumes of 0.05 N NaCl solution and continued with several changes of the same solution until the supernatant was clear. At this stage, no deterioration of the Sephadex has ever been noticed after frequent use for more than three months. The thin suspension was transferred to a large funnel, provided with a stirrer, and connected with a 2.0 x 30 cm. chromatography tube of glass fitted at the bottom with G - 1 filter and filled with 0.05N NaCl solution. When a bed of a few centimeters had formed above the G - 1 filter the stopcock at the bottom of the column tube was opened and Sephadex grains were allowed to settle under liquid flow. The final column measured 17 cm. in height. Then the columns could be washed with the M/15 phosphate buffer solutions desired (pH 6.8, ionic strength 0.26) without notable change of the outer dimensions of the bed.

Column II. —— 10 g. of DEAE-cellulose was suspended in 100 ml. of 0.1N NaOH, then transfered to a large vessel and diluted with water to 1 liter. After standing overnight, the cloudy supernatant liquid was decanted, and the sediment was washed 5 or 6 times by decantation to remove particles that would not settle. The final supernatant liquid was almost clear and was free of alkali. This adsorbent was poured as a slurry into a glass tube fitted at the bottom with G - 1 filter and was allowed to settle under flow conditions induced by gravity. After gravity settling was nearly complete, the adsorbent was further compacted by the application of air pressure until a constant column height was achieved. After such preparation, the column would not run dry under gravity flow.

The packed adsorbent column was then washed with several column volumes of the M/15 phosphate buffer to ensure pH and temperature equilibrium.

Approximately 3 g. of adsorbent was used to prepare packed columns of 25 x 1.2 cm. The exact height depended on the adsorbent employed.

Development of chromatogram:

The eqilibrated protein samples were washed into the column with several 1-ml. portions of buffer before the continuous flow of buffer was begun and slowly filtrated down.

For Sephadex column, the rate of the subsequent elution with the buffer usually adjusted 1 drop/7-10 sec. and the effluent fractions were collected every 3 g. by Toyo-Roshi automatic fraction cell collector (Model SF-200 A).

For DEAE-cellulose column, flow rates of approximately 6 ml./hr. were obtained with columns of the exchanger 25 cm. long and 1.2 cm. wide with a hydrostatic head of 60 to 90 cm. Fractions were collected every 3 g. by an automatic fraction collector. Elution was accomplished by changing the pH of the eluting buffer, except certain cases in which ionic strength was varied according the purpose. Two methods of elution were employed: a gradient elution (Peterson and Sober, 1956) and stepwise changes of the buffer. Separations were performed at cold room ($2^{o}$ C) unless otherwise noted. A variety of buffers was satisfactory or the necessary pH range (pH 5.9 to 9.2). Phosphate buffer of ionic strength 0.26 was used in general.

### Examination of effluent:

During purification of the inhibitor, protein concentrations were shown by an optical density at 280 mμ ($E_{280}$) in Beckman spectrophotometer (Model QR-50) with standard cells of 1 cm light path. Total nitrogen was determined by micro-kjeldahl analysis. Ammonium ion was tested by Nessler's reagent.

### Enzymes:

The enzymes used in this study, papain and crystalline trypsin were purchased from the Merck Co. Ltd., and Washington Biochemical Corporation. α-chymotrypsin was supplied by Eisai Co. Ltd. Proteolytic euglobulin was prepared from inflamed skin at maximum stage (24 hours) of Arthus reaction according to our previous (Hayashi et al. 1958; Udaka, 1960).

### Protease inhibitor assay:

Inhibitory effect of the inhibitor was determined by casein digestion according to Udaka (1960). Its effect was shown by activity of the inhibitor to inactivate one papain unit. Papain was prepared as 1.5 % suspension according to the previous method (Udaka, Harada and Hayashi, 1961). One papain unit was defined as the activity of papain giving digestion product with $1.0 \times 10^{-3}$ at $E_{276}$.

The potency was expressed as units of inhibitory activity per absorbency at 280 mμ ($IU/E_{280}$).

Inhibition of trypsin, $\alpha$-chymotrypsin and proteolytic euglobulin was also measured by the same method.

### Purification of inhibitor:

Unless otherwise stated, all purification steps were performed at $2^{\circ}$ C. Chromatography was performed on column of Sephadex G-50, prepared according to Porath and Flodin (1959), and of DEAE-cellulose, prepared according to Peterson and Sober (1956). Concentration of effluent fraction was performed according to Flodin, Gelotte and Porath (1960). The first step of the purification procedure was identical with the previous method (Hayashi et al. 1958; Matsubo, 1960; Udaka, 1960)

### Step 1. Extraction and fractionation of skin acetone powder.

Skin acetone powder (10 g.) was extracted with 10 volumes of M/15 phosphate buffer of pH 6.8 for 16 hr. and the suspension centrifuged at 5000 r.p.m. for 15 minutes. The supernatant fluid was filtered with Buchner funnel through one sheet of Toyo-Roshi filter paper No. 5C.

From the extracts euglobulin fraction was prepared by addition of saturated ammonium sulfate (50 ml. per dl.) according to Samuely and Strauss (1914).

### Step 2. Separation of ammonium sulfate from euglobulin fraction.

The euglobulin fraction, prepared from the skin extract, was suspended in M/15 phosphate buffer to give highest concentration. After centrifugation at 5000 r.p.m. for 15 minutes, the supernatant was diluted the same buffer to give an absorption at 280 m$\mu$ of between 18 and 22 and placed on a Sephadex column (2.0 x 17 cm.) which had been previously equilibrated with the same buffer and ionic strength 0.26. This equilibrated protein samples were washed into the column with several 1-ml. portions of buffer before the continuous flow of the buffer was begun and slowly filtrated. The filtration was made at a rate of 1 drop per 7-10 sec. and the effluent solution was collected every 3 g. by Toyo-Roshi's automatic fraction collector (Model SF-200A). The fractions were analyzed by ultraviolet light absorption at 280 m$\mu$. Ammonium ion was tested by Nessler's reagent. The chromatographic pattern of effluent solution is shown in Fig. 1.

Fig. 1

A qualitative analysis for ammonium sulfate ions in the first peak (peak 1) was negative, showing that the proteins had been freed effectively from salt. The second peak (peak 2) contains a low molecular weight substance with an absorption maximum at 262 mμ. The yield of material absorbing in ultraviolet at 280 mμ was about 97 % and its potency was about 8.

Step 3. Chromatography of inhibitory fraction on DEAE-cellulose

The clear filtrate (peak 1), obtained from the previous step, was diluted with M/15 phosphate buffer of pH 6.8 to give an absorption at 280 mμ of between 6.0 and 8.0 and dialyzed against the same buffer of pH 5.9 for 3 hours. It was applied to a column of DEAE-cellulose, 1.2 x 25 cm. which had previously been equilibrated with the same buffer. Elution was accomplished by pH stepwise changes (pH 5.9 to 9.2) of the eluting buffer at ionic strength 0.26. Flow rates was approximately 6 ml. per hour, and the effluent solutions were collected every 3 g. by an automatic fraction collector.

Fig. 2

As shown in Fig. 2, six major chromatographic components are obtained: the first comprised approximately 35 per cent, the second 7 per cent, the third 12 per cent, the fourth 9 per cent, the fifth 7 per cent and the sixth 5 per cent of the total protein recovered. The peak A contains highly active inhibitor; and the activity increases approximately 11 times as the original activity, about 8 $IU/E_{280}$. The yield of this fraction from DEAE-cellulose eluate is about 18 % and its potency is about 90 $IU/E_{280}$.

Step 4. Concentration of inhibitory fraction by Sephadex G-25.

To 80 ml. of the fraction (peak A), obtained from the previous step, 8 g. of Sephadex G-25 were added. The sieve fraction of this material was 50-100 mesh and its water regain was 2.4 g. per g. of dry Sephadex.

The thick suspension was stirred for 10 minutes to obtain complete swelling of the gel, after which the gel grains were separated from the concentrated inhibitor solution by centrifugation at 4000 r.p.m. for 30 minutes. The precipitates were then washed by spraying a small amount of water. This washing procedure was repeated again. The centrifugates and washes were pooled and subjected to an additional concentration step. After a fourth treatment the volume was reduced to an extent which made further purification. During concentration of the inhibitory fraction, the inhibitory activity against papain was measured following the method described above

<div align="center">Table I</div>

Experiments presented in Table I. illustrate that concentrated fraction contain  usually over 89 % of the total potency of original solution, and that each potency of the inhibitory fractions after 1, 2, 3 and 4 steps are shown as 130, 199, 331 and 582 $IU/E_{280}$. These results demonstrate that inhibitory activity increases approximately 6 times as the original activity, about 90 $IU/E_{280}$ and that the inhibitor is almost quantitatively recovered in the 8-fold concentrated solution.

## Step 5.  Crystalization

The concentrated inhibitory fraction with an activity of about 580 $IU/E_{280}$ was dialysed against M/15 phosphate buffer of pH 6.6 for 6 hours and filtrated through the G-3 glassfilter. The filtrate was allowed to stand at $2^o$ C for 2 to 5 days. Precipitation was complete during these days and fibrous precipitate obtained. The fibrous precipitates are shown in Fig. 3.

The activity of the fibrous precipitate was found at the range of 1150 and 1300 $IU/E_{280}$. Reprecipitation was carried out following the same manner as the first precipitation; slight increase in the activity of the precipitate was obtained after the reprecipitation. The extent of purification and the yield at various steps are ilustrated in Table II.

<div align="center">**Table II**</div>

## Effect of inhibitor on papain, trypsin and α-chymotrypsin:

Papain was prepared as a 1.5 % suspension according to previous the method (Udaka, Harada and Hayashi, 1961) and its filtrate used as enzyme solution. Trypsin was used in a concentration of 10 /ml. (Green and Work, 1953). α-chymotrypsin was used as a $5 \times 10^{-2}$ Ch.U. solution.

To 2.0 ml. of each enzyme solution, 2.0 ml. of inhibitor solution was added, and the homogeneous mixture was allowed to stand for 30 minutes at room temperature. The inhibitor mixture was used for determinations of the protease activity following the method described above.

## Fig. 4

Experiments presented in Fig. 4 indicate that this inhibitor has inactivates papain but has no effect on trypsin and α-chymotrypsin; these results are in agreement with our previously observations in the use of partially purified inhibitor (Udaka, Harada and Hayashi, 1961) ; and the inhibitor is different from naturally occurring trypsin inhibitor of polypeptide nature (Kunitz and Northrop, 1936; Schmitz, 1938).

Furthermore, this inhibitor was found still active when brought to $100^{o}$ C for 5 minutes, $80^{o}$ C for 30 minutes, or $-5^{o}$ C for 7 days.

### Effect of Inhibitor on Proteolytic Euglobulin:

Proteolytic euglobulin was prepared from inflamed skin at maximum stage (24 hours) of Arthus reaction according to the method (Hayashi et al., 1958; Udaka, 1960). The effect of the inhibitor on the euglobulin protease was measured following the method described above.

## Fig. 5

Experiments presented in Fig. 5 indicate the strong effect of the inhibitor in inactivating the euglobulin protease.

### Comparison of inhibitors newly and classically isolated;

The classical isolation procedure from the same source was described in previous paper (Hayashi et al., 1958; Matsuba, 1960; Udaka, 1960). The procedure on extraction and fractionation of skin acetone powder were identical with Step 1 described above.

To 10 ml. of euglobulin solution, suspended at the concentration of approximately 1.5 to 1.8 per cent by Hitachi's protein refractometer, 10 ml. of 6 per cent trichloroacetic acid was added, and the mixture allowed to stand at $37^o$ C. for 40 minutes until precipitate was complete. The mix mixture was centrifuged at 5000 r.p.m. for 15 minutes and filtered through one sheet of Toyo-Roshi filter paper No. 50. The filtrate was heated to $80^o$ C. for 5 minutes, cooled to $20^o$ C., and filtered. The second filtrate was dialyzed through cellophane membrane against ion-exchanged water, with frequent renewals, for 4 to 8 days at $2^o$ C. Precipitate in cellophane sack was collected and dissolved in saline.

During this procedure, the potency and the yield are compared with the newly isolated inhibitor. The activity of the inhibitor against papain was measured by the method described above.

## Table III

Experiments presented in Table III. show that the present purification procedure is more effective than the classical one. The ultraviolet spectrum of a solution of this newly isolated inhibitor showed the maximum absorbency at 276 mμ.

## DISCUSSION

Sephadex and exchanger such as DEAE-cellulose appear to have extensive possibilities for the analysis, preparation and purification of the labile substances of high molecular weight, such as protein. Sephadex was particularly valuable in the desalting phase of this study because of the easily and qualitatively separable effects for desalting and the high yields (Fig. 1 and Table III). In earlier experiments, as a desalting method, dialysis had usually been used for preparing euglobulin fraction. From those results there were shown that the yields of euglobulin after dialysis decrease about one-third of those separated by Sephadex G-50 (Table III). This indicates that denaturation of euglobulin may be protected effectively by Sephadex column chromatograph. Furthermore, this method presents many advantages; Porath and Flodin (1959), have found that this method causes

no complications corresponding to the clogging of dialysis membranes and that it is especially suited for changing a protein solution from one buffer system to another. Thus, this procedure is often necessary prior to ion exchange chromatography. However, in this procedure, the unavoidable dilution is naturally a disadvantage.

Sephadex was also very useful for concentrating proteins from diluted solution; the recovery was found more than 89 per cent at 8-fold concentration (Table I). This concerns the facts that Sephadex posses following properties : (1) it swells rapidly in water: (2) it is easily and quantitatively separable from the concentrate; (3) it is insoluble and not contaminate the concentrate; (4) it does not adsorb solutes which are to be concentrated. Recently several investigators have employed and recommended this concentrationg method for other substances sensitive to evaporation or freeze-drying, such as choline esterase, pancreatic lipase, phosphatase, phosphodiesterase (Flodin, Gelotte and Porath, 1960), and pepsin (Gelotte and Krantz, 1959).

DEAE-cellulose appears valuable in the purificatory phase in this study because of the comparatively large capacity for adsorbing proteins and sharp separable effects for elutions; and as shown in Fig. 2, the relatively high purities of the inhibitory fraction were obtained. This exchanger method appears superior compared to the boundary electrophoresis or the paper electrophoresis as an analytical method (Bain and Deutsch, 1947; Forsythe and Foster, 1950; Longsworth, Cannan and MacInnes, 1940), particularly for those constituents which have isoelectric points close to one another or which are present in small quantities. It has the advantage, however, of being time-consuming compared to the latter methods.

Following the method described in this paper, the specific protease inhibitor was isolated as fibrous precipitate from the healing Arthus skin site (Fig 3). This purified inhibitor is non-precipitable with 3 per cent trichloroacetic acid, heat-stable, non-dialyzable and insoluble in water but soluble in salt solution, and it gives a faint biuret test and shows a maximum at 276 mu in the ultraviolet spectrum.

This inhibitor inactivates strongly papain, but has no effect on trypsin and $\alpha$-chymotrypsin (Fig. 4), indicating that this is different from naturally occurring trypsin inhibitor of polypeptide nature. Furthermore, this inhibitor inactivates the proteolytic activity of euglobulin, prepared from the 24-hour lesion of Arthus-type inflammation (Fig. 5). Similar results have also been obtained with partially purified inhibitor prepared from the same source (Udaka, 1960). These results indicate that this inhibitor is identical the classically purified inhibitor and isolated in the form of more purified form by the present procedure. In vivo effects of this purified inhibitor will be described in a separate paper.

## SUMMARY

A specific protease inhibitor was isolated from the healing Arthus skin site and highly purified as fibrous precipitate. This purification procedure consisted of fractionation with $(NH_4)_2SO_4$, chromatography with Sephadex G-50 and DEAE-cellulose, and concentration with Sephadex G-25. This inhibitor inactivates certain SH-protease in the lesion of Arthus reaction and papain but has no effect on trypsin and $\alpha$-chymotrypsin.

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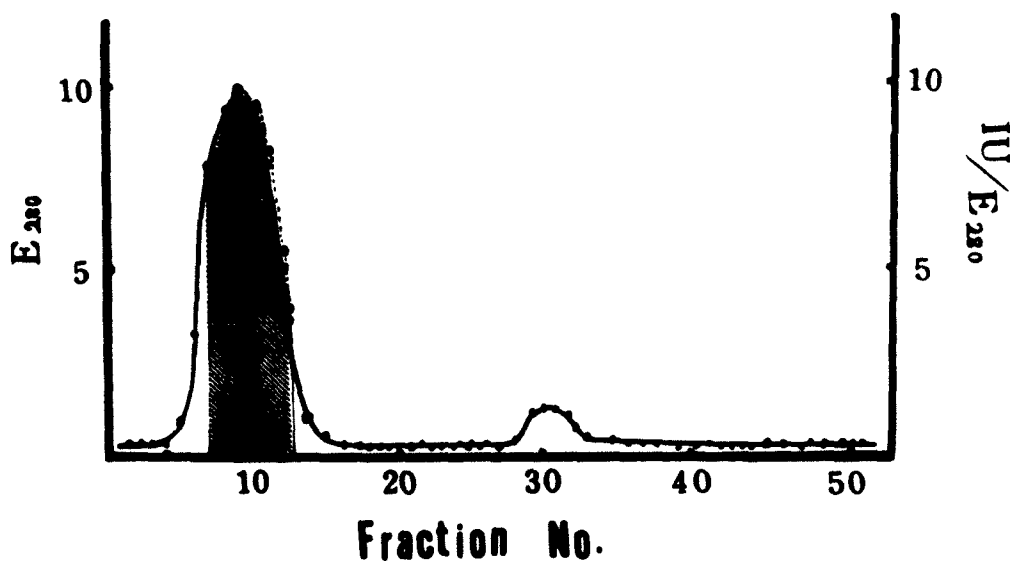
Fig. 1   Chromatographic Pattern of Inhibitory Euglobulin

   Fraction*

Column 2.0 x 17 cm. of Sephadex G-50 equilibrated with
starting buffer. Eluent was M/15 phosphate buffer of pH
6.8. Flow rate was 1 drop per 7 - 10 seconds. Fractions
were collected every 3 g. Abscissa: fraction number of
eluent; left ordinate: ——, absorbancy at E ; right
ordinate: —•——•—, inhibitory potency, $IU/E_{280}$. The shaded
area represents the fraction used for further purification.
* prepared from the healing Arthus skin site.
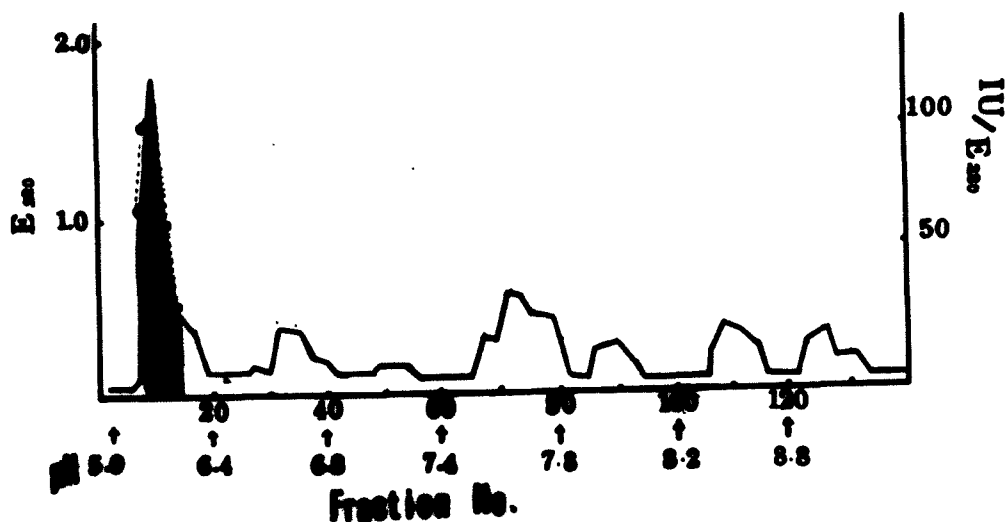
Fig. 2 Chromatographic Pattern of Inhibitory Fraction*

Column 1.2 x 25 cm. of DEAE-cellulose equilibrated with
starting buffer. Eluent was M/15 phosphate buffer of
indicated pH. Flow rate was about 5 ml. per hour. Fractions
were collected every 3 g. Abscissa: fraction number of
eluent; left ordinate: ————, absorbancy at $E_{280}$; right
ordinate: —•——•—, inhibitory potency, $IU/E_{280}$. The
shaded area represents the fraction used for further puri-
fication.
* prepared from Sephadex eluate.

TABLE I. Concentration of Inhibitory Fraction with Sephadex G-25

| | Inhibitory fractions | | Concentration | Potency | Yield |
|---|---|---|---|---|---|
| | In | Out | $E_{280}$ | $IU/E_{280}$ | |
| | ml. | ml. | | | % |
| Original sample* | 80 | | 1.4 | 90 | 100 |
| 1st. step | | | | | |
| main fraction | 80 | 53 | 2.0 | 130 | 97 |
| washed sample | 3 | 2.6 | 0.52 | | |
| washed sample | 3 | 2.8 | 0.08 | | |
| 2nd. step | | | | | |
| main fraction | 55 | 34 | 3.1 | 199 | 94 |
| washed sample | 3 | 3.1 | 0.98 | | |
| washed sample | 3 | 2.6 | 0.17 | | |
| 3rd. step | | | | | |
| main fraction | 37 | 21 | 4.7 | 331 | 93 |
| washed sample | 2 | 2.2 | 0.36 | | |
| washed sample | 2 | 1.7 | 0.11 | | |
| 4th. step | | | | | |
| main fraction | 19 | 11 | 7.9 | 582 | 89 |
| washed sample | 2 | 2.4 | 0.50 | | |
| washed sample | 2 | 2.1 | 0.44 | | |

* obtained from DEAE-cellulose elute of inhibitory fraction.

TABLE II. Extent of Purification of Euglobulin Fraction

| Fraction | Inhibitor Average Potency $IU/E_{280}$ | Yield % |
|---|---|---|
| Euglobulin ( from Sephadex eluate ) | 8 | 100 |
| Inhibitory fraction ( from DEAE-cellulose eluate ) | 90 | 18 |
| Concentrated fraction | 582 | 12 |
| Fibrous precipitate | 1260 | 3 |
| Reprecipitate | 1310 | 0.4 |
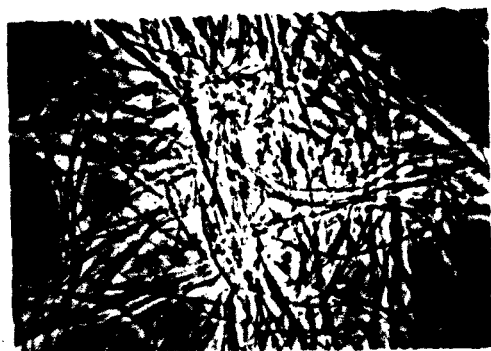


Fig. 3 A photograph shows the fibrous precipitate having inhibitory activity ( under phase contrast microscop, x 800).
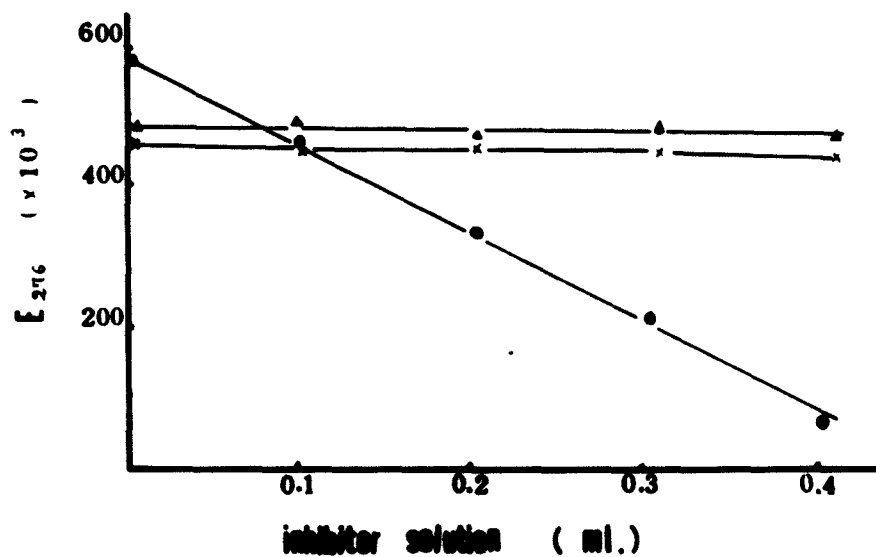
Fig. 4    Effect of Inhibitor on Papain, Trypsin, and
          ɑ-Chymotrypsin

Enzyme activity was measured by the casein digestion method:
0.5 % casein in M/20 phosphate buffer, pH 7.1, incubation
at 37°C for 30 minutes.  —•—•—; papain plus inhibitor.(0.10mg/ml).
—x—x—; trypsin plus inhibitor.   —▲—▲—; ɑ-chymotrypsin
plus inhibitor.
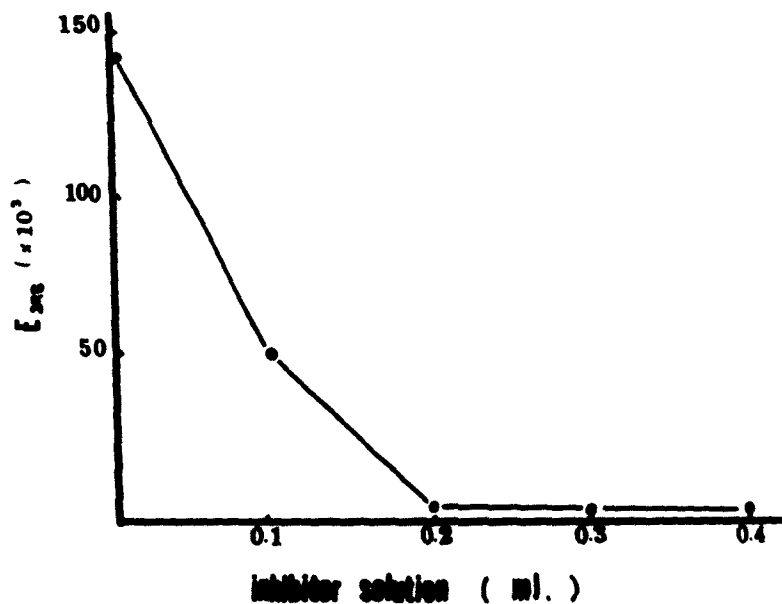
Fig. 5    Effect of Inhibitor on Proteolytic Euglobulin*

Enzyme activity was measured by the casein digestion method:
0.5 % casein in M/20 phosphate buffer, pH 7.1, ionic strength
0.25, incubation at 37°C. for 60 minutes. —•—•—; proteolytic
euglobulin( 0.97 mg.N/ml.) plus inhibitor ( 0.10 mg.N/ml.).
* prepared from inflamed skin at the maximum stage (24 hours)
of Arthus reaction.

TABLE III.    Comparison of Inhibitor Newly and Classically Isolated

| Newly Isolated | | Inhibitory Fraction | Classically Isolated | |
|---|---|---|---|---|
| Potency IU/mg.N | Total N mg. | | Total N mg. | Potency IU/mg.N |
| | 102 | Extract from 10 g. of skin Acetone Powder | 102 | |
| 50 ( 8 ) | 15.5 | Euglobulin by dialysis    Euglobulin by dialysis | 5.2 | 40 |
| 580 ( 90 ) | 3.1 | DEAE cellulose eluate | | |
| | | | Inhibitory 0.3-sub- | 2.3 | 80 |
| 3500 ( 582 ) | 2.6 | Concentrated fraction    fraction | | |
| 12000 (1260 - 1270) | 0.6 | Fibrous precipitate    Precipitate | 0.26 | 8000 |

Enzyme activity was measured by the casein digestion method: 0.5 % casein
in M/20 phosphate buffer, pH 7.1, ionic strength 0.25, incubation at 37 C
for 30 minutes.   Potency is expressed as units of inhibitory activity per
mg. N ( IU/mg.N ).   The value in parentheses represent potency ( IU/1280)
as shown Table II.

ENDOGENEOUS MECHANISM IN VASCULAR PERMEABILITY IN INFLAMMATION.

1. PERMEABILITY FACTOR AND INHIBITOR AFFECTING INCREASED VASCULAR
   PERMEABILITY IN CUTANEOUS ARTHUS REACTIONS AND THERMAL INJURY

The capillary wall is a barrier freely permeable to water and electrolytes but only very slightly permeable to plasma proteins. The term "increased vascular permeability" therefore refers usually to an alteration in the vascular wall leading to an accelerated rate of passage of plasma proteins into the extravascular tissues. The phenomenon is undoubtedly one of the cardinal features of acute inflammation, i.e., the local reaction of living tissue to injury.

The permeability changes have widely been investigated in the skin of experimental animals with vital dye, like pontamine sky blue, Evans blue, or trypan blue, circulating in their blood (Menkin, 1938; Rawson, 1942-43); and it has well been accepted that these dyes become attached to circulating plasma proteins, particularly the albumins, when given intravenously, and therefore their accumulation in the injured or treated skin sites indicates an exudation of plasma proteins.

As recently reviewed by Wilhelm (1962), however, the evaluation of experimental results in such tests often lacks precision, although there are some excellent attempts to demonstrate quantitatively the amount of accumulated dye in the injured or treated skin sites; for instance, in terms of "mean lesion diameter" (Miles and Wilhelm, 1955), or by comparing the colour intensity with a series of comparator cards (Lockett and Jarman, 1958). In the present study, the locally accumulated dye (pontamine sky blue) is estimated quantitatively by our successful extraction method, by which the dye in the skin is extracted almost absolutely (Nitta, Hayashi and Worimatsu 1963).

Previously proposed endogeneous candidates for the permeability changes are divided into two groups (Miles and Wilhelm, 1960); the first group includes the amines; histamine (Lewis, 1927) and 5-hydroxytryptamine or serotonin (Rowley and Benditt, 1956), and their liberators (see Paton, 1951); and the second group includes the proteases; globulin permeability factor (Mackay, Miles, Schachter and Wilhelm, 1953), and kallikrein (Frey and Kraut, 1928); and the products of proteolysis, the polypeptides like leukotaxine, (Menkin, 1938), bradykinin (Rocha e Silva, Beraldo and Rosenfeld, 1949) and kallidin (Werle, Gotze and Keppler, 1937).

All these agents undoubtedly increase vascular permeability in low concentrations. Except histamine, serotonin and leukotaxine, however, there is no direct evidence on the presence of other substances in inflammatory tissues or exudates. Furthermore, no conclusive evidence has hitherto been provided to establish that any of histamine, serotonin and leukotaxine is present in inflammatory tissues or exudates in suitable concentrations at times when permeability is increased as a result of tissue injury, or that the effect of these agents is no longer detectable when permeability is restored to normal. These two criteria are obviously essential if a substance found in inflammatory tissues or exudates is to be regarded as an intermediary in the production of altered vascular permeability. Although the above list shall be reasonably complete at the moment, there is no reason to suppose that other responsible substance does not remain to be discovered.

The present paper describes observations indicating that the permeability changes in the cutaneous Arthus reactions and thermal injury appear diphasic; an immediate response, being mediated by the substance susceptible for antihistamine, and a prolonged delayed response —— the major permeability event —— being concerned to a previously undescribed type of permeability factor insusceptible for antihistamine. The permeability factor in the delayed response is found in the pseudoglobulin; and its inhibitor in the euglobulin prepared from the inflamed skin.


## MATERIALS AND METHODS

### Experiment 1

Sensitization of animals. —— Male albino rabbits (2.0 ♦ 2.3 kg.) bred in our laboratory were used. On the 1st, 3rd, 5th, 7th and 9th days, 3 ml. of bovine plasma albumin (25 mg./ml. in physiological saline; Armour's product) was injected subcutaneously in the skin of the back of the trunk of the animals. On the 30th and 38th days, the antibody content of each animal's serum was determined by precipitin test (Hayashi, Tokuda and Udaka, 1960). In tubes 3 mm. diam., serial dilutions of serum were mixed with equal volumes (0.1 ml.) of antigen, and results read after 6 hr. at room temperature.

Only animals with titres of $2^6 - 2^7$ were used.

Induction of Arthus reactions. —— The flanks of sensitized animals were closely clipped with an electric hair clippers. After 24 hr. 0.1 ml. bovine plasma albumin (25 mg./ml. in saline) was injected intradermally in 2 - 3 sites, 5 cm. apart on each flank. The antigen induced Arthus. reactions which were almost equally intense at all the sites.

Macroscopically, the inflammatory reactions commenced in 1 - 2 hr., were maximal in 20 - 24 hr., and then declined in intensity. The inflammatory reactions usually disappeared in 11 - 14 days after injection. The animals were unanaesthetized throughout the experiments.

Induction of thermal injury. —— Male albino rabbits (2.5 - 3.0 kg.) and rats (260 - 330 g. ) were used. The flanks of these animals were very carefully clipped with an electric hair clippers and then with scissors. To avoid "chemical burning", any paste such as barium sulphide was not used.

A polished steel disc, 1.0 cm. diam., closing one end of a steel tube and maintained by circulating water at a stated temperature ($\pm 0.25^\circ$ ), was applied to the clipped skin of rabbits and rats following the method of Wilhelm and Mason (1960). The disc temperature was assumed to be that of the immediately adjacent water, recorded by a thermometer inserted through a rubber bung closing the other end of the tube. The apparatus is essentially a modification of that used by Sevitt (1949). The maximal heating applied to animals —— $56^\circ$ for 20 sec. to rabbits and $54^\circ$ for 20 sec. to rats —— was comfortably tolerated in each case by animals lightly held by hand. The heated skin neither blisters nor becomes necrotic. All the animals were unanaesthetized throughout the experiments.

Macroscopically, the inflammatory reactions (chiefly consisting of oedema) commenced in 20 - 30 min., were maximal in about 6 - 8 hr., and then declined in intensity. The heating was applied in 4 - 5 sites, 4 cm. apart on each flank, and induced reactions which were equally intense at all the sites.

Measurements of permeability effects. — Rabbits were given intravenously pontamine sky blue, 60 mg./kg. as a 5 per cent solution in 0.425 per cent saline; rats were given 60 mg./kg. as a 2.5 per cent solution in 0.6 per cent saline. Such animals are referred as "blued". The intravenous injections of dye were made at intervals varying from 0 min. to 48 hr. after challenge injection of antigen or application of heated disc. The animals were observed for the rapidity of appearance of the blue stain.

At 30 min. after intravenous dye, the animals mostly were sacrificed by bleeding from the carotid arteries, because the bluing at the inflamed skin sites reached maximal in 30 min. after intravenous dye.

The locally accumulated dye was extracted and estimated quantitatively as follows (Nitta, Hayashi and Norimatsu, 1963): After removing the fat, the skin (occasionally including the muscle) was cut into several pieces with scissors. The skin pieces were immediately placed in 10-fold volumes of mixture of absolute ethanol and Dioxane (1 : 1) at about 20 mm.Hg of vacuum at room temperature, until small bubbles from the skin ceased to occur; the course of this procedure required 20 - 30 min. After removing the solvents by centrifugation, the skin pieces were placed in an incubator of 37°. After the incubation of 20 min., the skin pieces became just rubbery in consistence, they being minced very easily and finely with scissors.

The skin was placed in 10-fold volumes of Dioxan for 1 hr. at room temperature, with constant stirring. After removing the Dioxane, the skin was placed in 10-fold volumes of mixture of chloroform and methanol (2 : 1) for 30 min. at room temperature, with constant stirring; and when absolute ethyl ether at ¼ volumes of the above mixture was further added, the skin was precipitated. Ten min. later, the solvents were discarded by means of centrifugation.

To the residual skin, 2.5 ml. of 30 per cent aqueous pyridine was added, and heated in a water bath of 80° for 30 min. - 1 hr. After centrifugation, the clear but coloured supernatant fluid was obtained; the supernatant fluid was then made up to 3 ml. volume for convenient spectrophotometric estimation with the aqueous pyridine.

If necessary, the last procedure with the pyridine was repeated until the blue colour of the skin completely disappeared; and the supernatant fluids all were pooled.

The Shimazu's spectrophotometer (QR-50) was used. The dye in the supernatant fluid was estimated at 620 mμ in cells with 1 cm. light path, and a final volume of 3 ml. The total amount of dye was computed from the total volumes of the supernatant fluid and a standard calibration curve at 620 mμ for graded concentrations of dye in 30 per cent aqueous pyridine. According to previous study (Nitta, Hayashi and Norimatsu, 1963), the method can recover successfully about 90 per cent of dye injected intradermally.

Experiment 2

Induction of Arthus reactions and thermal injury in rabbits. ---
The antigen (0.2 ml.) was given intradermally in 10 sites in an area 10 x 10 cm. of the clipped flanks of sensitized rabbits; the reactions produced at each site were almost equally intense. The heating at $56^{o}$ for 20 sec. was applied in 40 sites in an area 10 x 12 cm. of the clipped flanks of normal rabbits· the application of heating induced reactions which were equally intense in all sites.

Preparation of skin acetone powder. --- Inflamed skin was excised immediately after killing the animals. Fat and muscle were removed, the skin finely minced with scissors and immediately frozen at $-80^{o}$. The frozen skin was cut into slices about 50 μ thick with a freezing microtome and the slices (30 g.) placed in 1.l. of cold acetone of which the temperature then was allowed to rise to $2^{o}$. Dehydration was continued with 3 changes of acetone (1.l.), each bath of acetone being held with the skin in the order of 30, 10 and 10 min. period, and then removed by filtration with suction. The powdered skin was then air-dried in a hood to remove residual acetone and desicated in vacuo over P2 O5 for 12 hr. The technique was described by Udaka (1960).

Extraction and fractionation for permeability factor. --- All procedures were carried out at 1 - $2^{o}$. Skin acetone powder was extracted for 4 hr. with 7-fold volumes of M/15 phosphate buffer, pH 7.4; the suspension centrifuged at 5000 r.p.m. for 15 min.; and supernatant fluid filtered through Toyo-Roshi paper No. 5C.

Unless stated in particular, the above phosphate buffer was used throughout the experiments.

From the extracts 3 preparations containing euglobulin, albumin or pseudoglobulin were fractionated with ammonium sulphate (Hayashi, Hattori, Mitsui and Takouchi, 1959). The protein fractions were dissolved for use in the buffer.

Chromatography of extracts and protein fractions. ─── Chromatography was performed on a column of Sephadex G-50 (Pharmacia's product, Uppsala) essentially according to Porath and Flodin (1959). All procedures were carried out at 1 - 2°.

The skin extracts were diluted with the buffer to give an absorbency 36 - 42 at 280 mμ ; and 3 ml. of each extract was eluted through the column (2.0 x 17.0 cm.) which had previously been equibrated with the buffer. The filtration was made at a rate of 5 drops/min. and fractions collected every 3.0 g. with the Toyo-Roshi automatic fraction collector (SF-200A); and effluent fractions each were analysed particularly by ultraviolet light absorption at 280 mμ.

On the other hand, 3 preparations containing euglobulin, albumin or pseudoglobulin were dissolved in the buffer to give an absorbency 21 - 24 at 280 mμ, except some special cases; after centrifugation at 5000 r.p.m. for 15 min., 3 ml. of each protein solution was eluted through the column in the same manner.

Protease assay. ─── This was performed according to Martin and Axelrod (1957) and Udaka (1960). One ml. of 2 per cent casein solution and 1 ml. buffer were mixed and held at 37° for 5 min. before adding 1 ml. of protein solution and 1 ml. of 0.15 M saline. Proteolytic activity was tested at various pH conditions (6.6 - 8.2) with the same ionic strength (0.25). The buffer concentration was 0.05 M.

After incubation at 37° for 60 min., 5 ml. of 6 per cent trichloroacetic acid was added. In control preparations which were not incubated, the protein solution was added after the trichloroacetic acid. The optical density at 276 mμ of the filtrate was estimated with the Shimazu's spectrophotometer (QR-50), using the filtrates from the control preparations as blanks.

## Experiment 1. Permeability changes in inflammation

Permeability changes in Arthus reactions. --- At sensitised skin sites injected with 0.1 ml. antigen, the permeability response consisted of two distinct phases: immediate --- appearing rapid, transient (lasting less than 30 min.) and less intense, and suppressed by local antihistamine; and delayed --- appearing more intense and prolonged (beginning in 1 hr., reaching its maximum in 4 - 5 hr. and disappearing in 24 hr.), and unaffected by local antihistamine.

### The immediate response

The bluing of the treated sites began after a few seconds of antigen injection. The blue colour rapidly increased in intensity and its maximal intensity was found in 5 min. old lesion (Fig. I). In unblued animals, there has not yet been observed any macroscopic reaction, except the bulla due to the injection itself. The response was suppressed almost completely when 0.1 ml. triprolidine maleate (100 $\mu$g./ml. in saline, Burroughs Wellcome's product) was injected intradermally together with antigen or 5 min. before antigen. No suppression of the bluing was observed with saline.

Apparently less intense, occasionally negligible, bluing was induced when the antigen was injected in the non-sensitized skin; the bluing was also suppressed completely by local triprolidine; and in the non-sensitized skin, any type of delayed response was not observed during 48 hr. of observation.

The interpretation of the local effects of antihistamine, including triprolidine (Green, 1953) is somewhat complicated by their non-antihistaminic side-actions, e.g., anticholinergic and anaesthetic effects. Triprolidine used in this experiment, however, has been shown to have an effect sufficiently specific to justify the use in investigating permeability response in injury (Wilholm and Mason, 1960); the drug appeared to be the best compound available for such work.

### The delayed response

The immediate response subsided in about 10 min.; and the delayed response began in about 1 hr., reaching its maximum in 4 - 5 hr., and decling thereafter (Fig. I).

The local oedema and erythema, in unblued animals, became visible in 1 - 2 hr., rapidly increasing in intensity, and reaching its maximum in 20 - 24 hr. Accordingly, the most important flow of blue was noted at 4 - 5 hr. old lesion, and therefore the vascular permeability changes in the Arthus reactions proceeded the maximal height of oedema and erythema.

The delayed response was elicited more intensely and regularly than the immediate response. However, very occasionally, the through between the two responses appeared very shallow or even negligible if the delayed response was provoked too rapidly.

The delayed response was nearly unaffected by local triprolidine (10 µg), given together with antigen, or during the maturation of the response, repeatedly; and special interest arose from such an insusceptibility to antihistamine of the permeability factor responsible for the delayed response.

Permeability changes in thermal injury. --- The heating was comfortably applied to animals --- $56^{\circ}$ for 20 sec, to rabbits and $54^{\circ}$ for 20 sec. to rats --- following the method of Wilhelm and Mason (1960). The heating at $60^{\circ}$ for 10 sec. (Sevitt, 1958) seemed unsuitable for the present purpose because of too pronounced lesion locally induced.

As observed in Arthus reactions, the permeability changes in thermal injury also appeared diphasic in rabbits and rats (Fig. II); immediate--- less intense and only transient (lasting less than 10 min.), and suppressed by local triprolidine (10 µg.); and delayed --- more intense and prolonged (beginning in about 1 hr. and disappearing in 6 - 8 hr.), and unaffected by local triprolidine (10 µg.), given before or during the maturation of the response; the general pattern of delayed response was not modified by antihistamine.

The immediate response in both species, particularly in rats, was irregular in appearance, occasionally being negligible or absent. The maximal intensity of the delayed response was found at 2 hr. old lesion for rabbits; and 3 hr. old lesion for rats. The observations were essentially in accordance with those of Wilhelm and Mason (1960).

## Experiment 2. Endogenous factor and its inhibitor affecting the delayed response

**Reproduction by skin extracts of permeability changes in Arthus reactions.** --- Each of the skin extracts, prepared at various intervals of Arthus reactions, was injected intradermally in the clipped flanks of rabbits --- extract (0.1 ml.) plus buffer (0.01 ml.) in the right flank; extract (0.1 ml.) plus triprolidine (10 μg. in 0.01 ml. buffer) in the left. Each animal's serum was taken at various intervals of Arthus reactions; and 0.1 ml. of each serum was given intradermally in the right flank. Intravenous dye was administrated immediately after intradermal injections, the animals mostly being killed 1 hr. later.

The bluing of the extract-injected sites began after a few minutes of dye injection, increasing rapidly in intensity, and reaching its maximum in 50 - 60 min. with the development of oedema.

The results recorded in Fig. III suggest that the time-course of permeability changes in the Arthus reactions (see Fig. I) is reproduced considerably well by the skin extracts prepared; and it shows that the extraction is reasonable in isolating the permeability factor affecting the delayed response in Arthus reactions. The permeability effects of the extract of lesion showing most intense delayed response (i.e. 4½ hr. old lesion), were most active but unaffected by local triprolidine: in contrast, those of the extract of the immediate response being less intensive but strongly suppressed by local triprolidine.

Negative results were obtained with all the sera from rabbits with Arthus reactions; this may suggest the absence of active permeability factor of its too low concentration in the serum.

**Demonstration of permeability factor in the skin extract.** --- The skin extract, prepared from 4½ hr. old Arthus lesion, was diluted with the buffer to give an absorbency 36 - 42 at 280 mμ; and 3 ml. of the extract was eluted through Sephadex column. Effluent fractions each were injected intradermally in the flanks of rabbits --- fraction (0.1 ml.) plus triprolidine (10 μg. in 0.01 ml. buffer) in the right; fraction (0.1 ml.) plus buffer (0.01 ml.) in the left.

Intravenous dye was given immediately after the injections, the animals mostly being killed 1 hr. later.

The results recorded in Fig. IV indicate that the permeability factor of the delayed response is contained only in the effluent fractions of the first group showing a definite absorption at 280 mμ. Such a spectrophotometric assay at 280 mμ promised a useful convenience in elucidating the location of permeability factor. The bluing at the treated skin sites, as observed with the original extracts, began after a few minutes of dye injection, increasing rapidly in intensity, and reaching its maximum in 50 - 60 min. with the development of mild oedema. The permeability effects f each fraction were not affected by local triprolidine.

Demonstration of permeability factor in pseudoglobulin. --- Three preparations containing euglobulin, pseudoglobulin or albumin, prepared from the extracts of 4½ hr. old Arthus lesion, were respectively diluted with the buffer to give an absorbancy 21 - 24 at 280 mμ, except special cases; 3 ml. of each protein solution was eluted through Sephadex column.

Each 0.1 ml. of effluent fractions showing positive absorption at 280 mμ was intradermally injected in the clipped skin. Intravenous dye was given immediately after the injections; the animals mostly being killed 1 hr. later.

The result recorded in Fig. V indicate that the permeability factor of the original extracts is predominantly concentrated in the pseudoglobulin, and the permeability effect of effluent fractions being intensive in parallel with the absorbancy at 280 mμ. The most active fraction was fraction No. 8. The bluing at the sites injected with the active fractions, began after a few minutes of dye injection, and reached its maximum in 45 - 60 min. with the development of oedema.

The permeability effect of the albumin was apparently less active, that of the euglobulin being most mild.

Properties of permeability factor of pseudoglobulin. --- The following assays all were performed on the above fraction No.8 which is named PF-fraction. All the materials were given intradermally in the flanks of rabbits.

Intravenous dye was administrated immediately after the injections, the animals mostly being killed 1 hr. later.

(a) **Effect of triprolidine.** —— PF-fraction (0.1 ml.) plus buffer (0.01 ml.) in the right; the fraction (0.1 ml.) plus 10 μg. triprolidine in the left.

No suppression of permeability effect of PF-fraction was found with local triprolidine (Table I).

(b) **Effect of heating.** —— PF-fraction was heated in a water bath of $60°$ for 30 min., $75°$ for 30 min., or $100°$ for 5 min.; after centrifugation at 5000 r.p.m. for 15 min., supernatant fluids each being tested —— original PF-fraction (0.1 ml.) in the right flank; heated fraction (0.1 ml.) in the left.

The permeability factor of PF-fraction seemed thermolabile; its activity was almost completely lost by heating to $60°$ for 30 min. and complete loss of activity was obtained by heating to $75°$ for 30 min., or $100°$ for 5 min. (Table I and Fig. VI).

(c) **Effect of dialysis.** —— PF-fraction was dialysed through a cellophane membrane (Visking's seamless cellulose tube) against the buffer at $1-2°$ for 24 hrs —— remaining fluid (0.1 ml.) in the right flank; original fraction (0.1 ml.; held at $1-2°$ for 24 hrs.) in the left. During the dialysis, there was not observed any precipitate.

The permeability factor of PF-fraction seemed non-diffusible; no effect of dialysis was found for the permeability factor (Table I).

(d) **Effect of soy bean trypsin inhibitor (SBTI).** —— PF-fraction was mixed with various concentrations of highly purified SBTI (15, 20, 50 and 60 μg./ml. in saline; Novo's product, Norway) in a ratio of 1 and 1: the mixtures each being held for 30-50 min. at room temperature —— mixture (0.1 ml.) in the right flank; PF-saline mixture (0.1 ml.) in the left. This was performed essentially according to Miles and Wilhelm (1960).

No suppression of permeability effect of PF-fraction was found with various concentrations of local SBTI (Table I and Fig. VI).

(e) **Effect of di-iso-propyl fluorophosphonate (DFP).** —— PF-fraction was mixed with $10^{-5}$, $10^{-4}$, or $10^{-3}$ M DFP (diluted with saline) in a ratio of 1 and 1; the mixtures each being held for 1, 2 or 4 hr. at room temperature —— mixture (0.1 ml.) in the right flank; PF-saline mixture (0.1 ml.) in the left. This was also performed essentially according to Miles and Wilhelm (1960).

The results shown in Table I and Fig. VI indicate that DFP has no effect on the permeability factor of PF-fraction.

(f) **Effect on casein digestion.** — One ml. of 2 per cent casein solution and 1 ml. buffer were mixed and held at 37° for 5 min. before adding 1 ml. of PF-fraction and 1 ml. of 0.15 M saline. The reaction mixtures were maintained at pH 7.1, buffer concentration of 0.05 M, and ionic strength 0.25. After incubation at 37° for 60 min., 5 ml. of 6 per cent trichloroacetic acid was added. The optical density at 276 mu of the filtrate was estimate with the Shimasu's spectrophotometer.

As shown in Table I, no caseinolytic activity is detected with PF-fraction inspite of its high permeability effect. Such negative results with the PF-fraction were in accordance of our previous observations that the local proteolytic (caseinolytic and fibrinolytic) activity in Arthus reactions was found only in the euglobulin prepared but not in the pseudoglobulin and albumin fractions ( Hayashi, 1956, 1958; Hayashi et al., 1958; Udaka, 1960, 1962).

(g) **Effect on extravascular migration of leucocytic cells.** — PF-fraction (0.1-0.2 ml.) injected intradermally in the right flank; leukotaxine (0.1-0.2 ml.; 5 mg./ml. in buffer; see below) in the left. After 45-90 min., the skin sites were excised and fixed in 10 per cent neutral formalin for microscopic examination of sections.

In contrast to the striking effect of leukotaxine on extravascular migration of polymorphonuclear leucocytes (Menkin, 1940), the PF-fraction showed no such an effect (Fig. VII). However, 0.1 ml. of the PF-fraction can induce the permeability changes more intensive than the same volume of leukotaxine can (Table III).

(h) **Effect on contraction of smooth muscle.** — Following the usual way the preparations of guinea-pig ileum were suspended in Tyrode solution in an 20 ml. bath and kept at 37°. Various volumes (0.1, 0.5 or 1.0 ml.) of PF-fraction were added to the bath; 0.1 ml. of bradykinin (0.5 µg./ml.; see Table III) was tested as the control.

No contraction of the ileum was observed with any volume of PF-fraction; in contrast, distinct contraction observed with 0.1 ml. of bradykinin solution. However, 0.1 ml. of the PF-fraction was more active than the same volume of the bradykinin solution in inducing increased vascular permeability.

<u>Comparison of permeability effects of PF-fractions isolated at various</u>
<u>intervals of Arthus reactions</u>. --- Permeability effects of PF-fractions,
isolated from various intervals of Arthus lesions, 30 min., 4½ and 12 hr.
old, were compared· the test was performed on the almost same concentration
(absorbency 10.0 - 11.0 at 280 mμ) of PF-fractions; 0.1 ml. of each
fraction injected intradermally in the flanks of rabbits. Intravenous dye
was given immediately after the injections; the animals mostly being
killed 1 hr. later.

The results recorded in Table II indicate that the permeability effects
of PF-fractions clearly run parallel to the time-course of permeability
changes in Arthus reactions (c.f. Fig. I). The observations may give a
reasonable support to regard the permeability factor as an intermediary
or one of the intermediaries for the delayed response in Arthus reactions.

<u>Demonstration of anti-permeability factor in euglobulin</u>.--- As shown
in Fig. I, permeability changes in Arthus reactions become apparently less
intense in 12 hr. old lesion, suggesting the local appearance of anti-
factor or destruction of the permeability factor. Three preparations
containing euglobulin, pseudoglobulin or albumin, prepared from 12 hr. old
Arthus lesion, were diluted with buffer to give an absorbency 15-20 at
280 mμ; 10 ml. of each protein solution was eluted through Sephadex
column: PF-fraction was mixed with effluent fractions, negative with
Nessler's, in a ratio of 1 and 1; and before injections each mixture was
held for 30 min. at room temperature . --- Mixture (0.1 ml.) in the right
flank; PF-buffer mixture (0.1 ml.) in the left. Intravenous dye was given
immediately after the injections, the animals mostly being killed 1 hr.
later.

The results recorded in Fig. VIII indicate that the anti-factor for the
permeability factor of PF-fraction is predominantly concentrated in the
euglobulin; and the effect is found located in certain effluent fractions,
particularly in fraction No.15, showing a lower absorbency at 280 mμ.
This fraction is called anti-PF-fraction.

<u>Effect of anti-factor of euglobulin on various permeability factors</u>.---
Effect of the above anti-PF-fraction was tested on the following permea-
bility factors;

**Leukotaxine.** ---- This substance (M-acetic fraction) was isolated by the method of Menkin (1960) from cell-free exudate of turpentine-induced pleuritis of dogs; the amorphous powder was suspended in buffer in a concentration of 5 mg./ml. and centrifuged.

**Bradykinin.** ---- Synthetic bradykinin (Sandoz's product, Basle) was dissolved in buffer in a concentration of 1.0 µg./ml.

**Kallidin.** ---- Pure kallidin was dissolved in buffer in a concentration of 2.0 unit/ml.

**Globulin permeability factor.** ---- Highly purified factor of rabbit's serum was dissolved in buffer in a concentration of 3 mg./ml.

**Kallikrein.** ---- Kallikrein (Bayer's product, Leverkusen) was dissolved in buffer in a concentration of 0.1 unit/ml.

The above listed solutions, and PF-fractions of Arthus lesion and thermal injury (see below) each were mixed with the anti-PF-fraction in a ratio of 1 and 1. After standing for 30 min. at room temperature, each mixture (0.1 ml.) was injected intradermally in the right flank; PF-buffer mixture (0.1 ml.) in the left. Intravenous dye was administrated immediately after the injections, the animals mostly being killed 1-2 hr. later.

The results recorded in Table III indicate that the anti-factor of euglobulin can suppress considerably the effects of Arthus- and thermal permeability factors, but has no effect on leukotaxine, bradykinin, kallidin, globulin permeability factor and kallikrein; these observations suggest that the inflammatory PF found in this experiment may be different from various candidates as listed above.

Furthermore, the anti-factor was found still active when brought to 60° for 30 min., or 75° for 15 min.

Previous experiments in this laboratory showed that the activity of certain skin SH-protease increased in parallel with the development of Arthus reactions; and that inactivation of the protease by the polypeptide inhibitor may be correlated with the subsequent and gradual cessation of the reactions (Hayashi, 1955, 1956; Hayashi et al., 1958; Udaka, 1960, 1962; Matsuba, 1960; Ito, 1960). The protease and anti-protease each were released by tissue phagocytes during antigen-antibody reaction, the enzyme being released before its inhibitor (Hayashi, Tokuda and Udaka, 1960; Tokuda, Hayashi and Matsuba, 1960).

Both substances were recovered in ouglobulin fractions. More recently, the anti-protease was highly purified by means of column chromatograph (Hayashi, 1962; Udaka, Koono and Hayashi, 1962).

The purified anti-protease was dissolved in buffer at various concentrations (0.4 - 0.8 mg.N/ml.) capable of inactivating high activity of the SH-protease. PF-fraction from Arthus- and thermal lesion was mixed with the anti-protease in a ratio of 1 and 1, and the mixtures each were held for 30 min. at room temperature ——mixture (0.1 ml.) in the right flank; PF-saline mixture (0.1 ml.) in the left.

As shown in Table III, the anti-protease has no effect on the permeability factors of Arthus- and thermal lesion; the observations indicate that the anti-PF of ouglobulin is different from the anti-protease similarly contained in the ouglobulin. In turn, it is evident that our inflammatory PF is distinct from the skin SH-protease.

Endogenous factor affecting delayed response in thermal injury. ——

The same type of experiment was carried out on various samples from thermal injury in rabbits. As shown in Fig. IX, the permeability factor affecting the most intense delayed response, i.e., 2 hr. old lesion, was predominantly concentrated in the pseudoglobulin; and its effect of effluent fractions being in general most active in fraction No.8, showing a highest absorption at 280 mμ.

Properties of the permeability factor were assayed following the same manner as noted above; and it was clarified that this factor was indistinguishable at the moment from that of Arthus reactions, because it also was thermolabile, non-diffusible and insusceptible to triprolidine, SBTI, DFP and our anti-protease. This factor had no effect on stimulation of smooth muscle of ileum of guinea-pigs or extravascular migration of leucocytes.

Following the same method as described above, the anti-substance for the permeability factor was found in the ouglobulin prepared from the 8 hr. old lesion. This anti-factor was also eluted into the fraction No.15 (Table III).

# DISCUSSION

In the present experiment, leakage of circulating dye (pontamine sky blue) into the inflamed skin sites was shown quantitatively by the amounts of dye extracted from the sites following our method (Nitta, Hayashi and Norimatsu, 1963). Application of such successful technique in the test of increased vascular permeability promised a precise evaluation of experimental results.

In two distinct types of inflammation, i.e., Arthus reactions and thermal injury, the permeability changes consisted of two different phases --- immediate and delayed. Although there was small variation in the pattern and duration according to the nature of injury, the delayed response --- the major permeability event --- was essentially a characteristic feature, common to such experimental injuries. In contrast, the immediate response was the minor permeability event, it being only transient. Similar observations on permeability changes in inflammation have been reported for example, thermal injury (Sevitt, 1958; Wilhelm and Mason, 1960; Spector and Willoughby, 1959; Allison and Lancaster, 1959); bacterial infections (Burk and Miles, 1958; Petri et al., 1952; Miles and Wilhelm, unpublished; Elder and Miles, 1957; MacFarlane and Knight, 1941); ultrasonic injury (Burk; see Wilhelm, 1962); traumatic and chemical injury (see Wilhelm, 1962).

It seems very probable that the immediate response is mediated by the substance susceptible to local triprolidine, possibly histamine. The conclusion was also drawn from the observations of Wilhelm and Mason (1960) in thermal injury. Special interest therefore arises from the insusceptibility of the delayed response to local triprolidine. No satisfactory mediators for the delayed response have been obtained in any type of tissue injuries.

The permeability factor was isolated from the Arthus lesion showing delayed response, i.e., 4½ and 12 hr. old. This inflammatory PF is predominantly concentrated in the pseudoglobulin; and its effect of effluent fractions (through Sephadex column) of the pseudoglobulin being active in parallel with the absorbancy at 280 mμ.

At the moment this PF appears to be of protein nature but different from the proteases (globulin permeability factor and kallikrein) and the polypeptides (leukotaxine, bradykinin and kallidin) --- it may be distinguishable from these polypeptides by its thermolability, non-diffusibility and inability to stimulate smooth muscle or to induce an accumulation of leucocytes in the injected skin sites; it resembles globulin permeability factor and kallikrein rather than the polypeptides but it is clearly insusceptible to SBTI and DFP which can inactivate the effects of globulin permeability factor and kallikrein. The proteases and our inflammatory PF have no caseinolytic activity. This PF is distinct from histamine by its insusceptibility to triprolidine.

More reasonable evidence was obtained by the use of our anti-PF of the euglobulin, isolated from Arthus lesion 12 hr. old; this substance can suppress the effects of the PF but has no effect on the polypeptides and proteases. Since this anti-PF is still active when brought to 60° for 30 min., or 75° for 15 min, it may be distinguishable from the inflammatory SH-protease (Hayashi, 1956; Hayashi et al., 1958; Udaka, 1960, 1962) whose activity is lost almost completely by heating to 60° for 10 min. The anti-PF is also distinct from our anti-protease, because use the latter polypeptide has no effect on the PF.

The delayed response in Arthus reactions seems explicable in terms of our inflammatory PF, since the effect of the PF was more active at times when permeability changes was increased; its effect becoming less active as permeability changes subsided. This criteria seems essential if this substance with higher permeability effect is to be regarded as an intermediary in the vascular phenomenon. The question whether the balance between the inflammatory PF and its inhibitor determines the intensity, extent and duration of the delayed response --- the major event in the vascular phenomenon --- in Arthus reactions, waits further study. Further purification of both substances is now in good progress; the results will be described in a separate paper.

Recent tissue culture experiments showed that tissue phagocytes, i.e., tissue-monocytes and fibroblasts, submitted to the antigen-antibody reaction, released two distinct permeability factors --- the one susceptible to triprolidine; the other insusceptible to triprolidine, being released later. The latter PF closely resembled the inflammatory PF, because it was insusceptible to SBTI and DFP but affected by our anti-PF of euglobulin (Hayashi, 1962; Kinuwaki and Takaba, unpublished). Negative results with the anti-protease indicate that this cellular PF is distinct from the protease (SH-dependent), released from the cells during the antigen-antibody reaction (Hayashi, Tokuda and Udaka, 1960; Tokuda, 1959). Basic morphological changes occuring the antigen-antibody reaction have widely been studied in the above cultured cells (Hayashi and Ono, 1953, 1955; Hayashi, Ono and Matsumoto, 1955· Ono, 1958; Kuze, 1960; Takaba, unpublished).

At the moment we suppose that permeability changes in the delayed response of Arthus reactions may be associated with the cellular PF, released during the antigen-antibody reaction, in part, since each serum from rabbits bearing Arthus reactions showed no permeability effect. However, this not always deny the role of the serum proteases (globulin permeability factor and kallikrein) or polypeptides (bradykinin and kallidin) in the vascular phenomenon. There are many reviews concerning the biological significance of globulin permeability factor (Miles and Wilhelm, 1960; Miles, 1959; Wilhelm, 1962· Spector, 1958) kallikrein (Werle, 1960; Forell, 1960), bradykinin (Rocha e Silva, 1960; Elliott, 1961; Lewis, 1960; Schachter, 1960) and kallidin (Werle, 1960; Lewis, 1960). Concerning the essential role of our inflammatory PF, we must wait further results.

Similar inflammatory PF and its anti-PF are isolated from thermal injury; they are at the moment indistinguishable from the PF and its anti-PF of Arthus reactions. However, the considerable difference in the effects of both anti-PFs seems open the way to verify the essential difference in both PFs. It seems convinced that inflammation probably has many mediators including permeability factors, that differ according to the nature of the injury.

# SUMMARY

The time-course and mediation of increased vascular permeability in
Arthus reactions and thermal injury were investigated in the skin of
unanaesthetized rabbits and rats with pontamine sky blue circulating in
their blood. The locally accumulated dye was quantitatively estimated by
our extraction method.

1. The general pattern of vascular response is diphasic in these
species and injuries —— immediate and delayed. The temporary immediate
response is mediated by a substance susceptible to antihistamine (triproli-
dine), i.e., possibly histamine. The prolonged delayed response —— the
major event in vascular permeability —— is possibly mediated by a
previously undescribed substance, insusceptible to triprolidine: this
this factor is concentrated predominantly in the pseudoglobulin fraction,
isolated from Arthus and thermal lesion eliciting the delayed response.

2. This inflammatory PF, possibly protein in nature, is thermolabile,
non-diffusible, insusceptible to antihistamine, soy bean trypsin inhibitor
and di-iso-propyl phosphofluoridate, and fails to stimulate smooth muscle,
or to induce an extravascular migration of leucocytes, and has no caseinoly-
tic action. The PF seems different from the proteases (globulin permea-
bility factor, kallikrein and skin SH-protease) and polypeptides
(leukotaxine, bradykinin and kallidin). At the moment both PFs are indis-
tinguishable.

3. The effect of the inflammatory PF is active in parallel with the
time-course of permeability changes in the injury, suggesting the possible
role of this PF as an intermediary for the delayed response.

4. The anti-factor is found predominantly in the euglobulin fraction,
isolated from Arthus- and thermal lesion with decreased vascular response;
it can suppress the effects of both inflammatory PFs but has no effects of
the above proteases and polypeptides. This anti-factor is different from
the anti-protease of polypeptide nature, similarly contained in the
euglobulin fraction. The activity of anti-factor is still remained
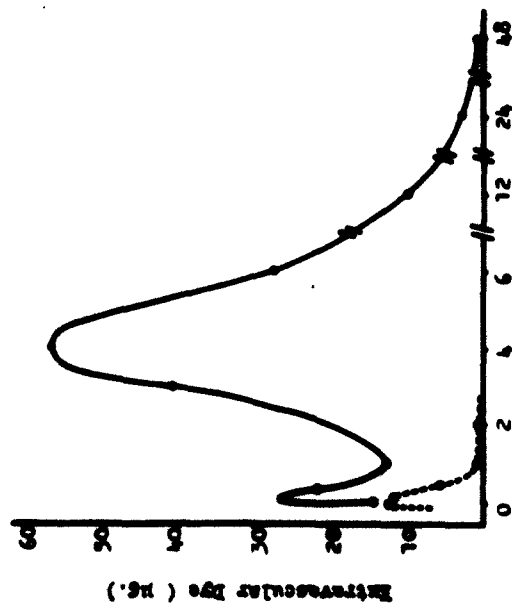unchanged after heating at $60^0$ for 30 min., or $75^0$ for 15 min.

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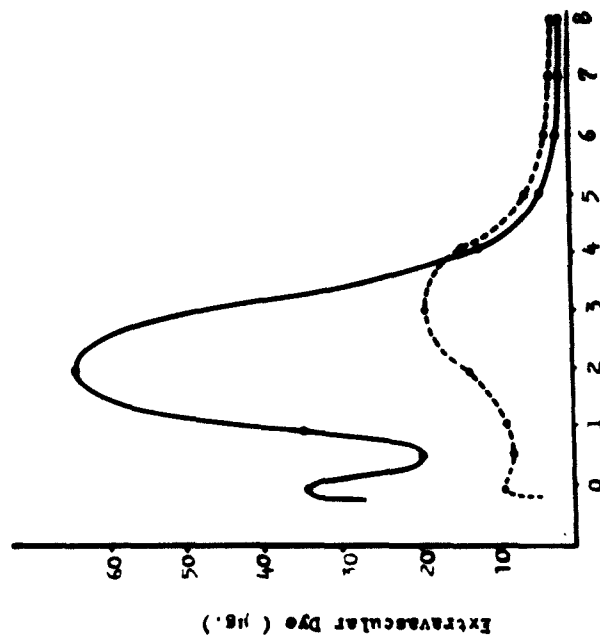    Vessels, ed. by M. Schachter, p. 232, Pergamon Press, Oxford.

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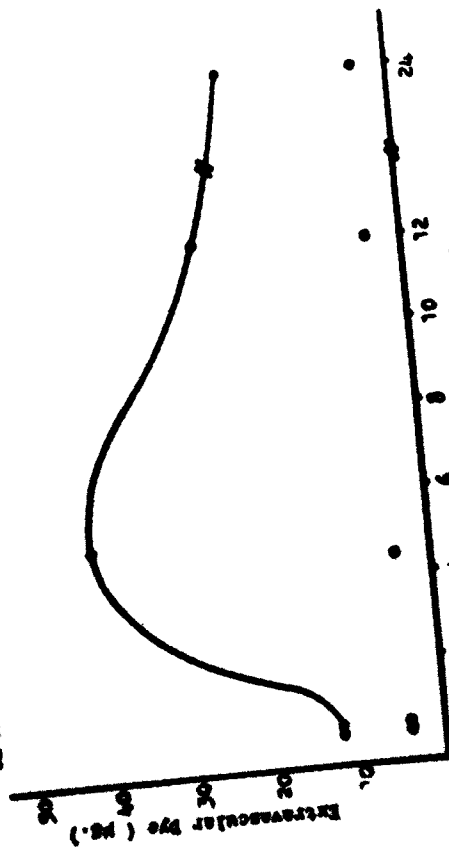
Fig. 2  Time-Course of Permeability Changes in Thermal Injury



Extravascular Dye ( µg.)

Age of Reaction at Permeability Test ( hrs.)

Rabbit, 56 C for 20 sec.
Rat, 54 C for 20 sec.

Fig. 1  Time-Course of Permeability Changes in Arthus Reaction



Extravascular Dye ( µg.)

Age of Reaction at Permeability Test ( hrs.)

2.5 mg. BSA to sensitized skin.
2.5 mg. BSA to non-sensitized skin.

Extravascular Dye ( ug. )

Fig. 5 Demonstration of Permeability Factor in Pseudoglobulin Eluted Through Sephadex Column

Fraction Numbers

Optical density
Extravascular dye

Optical Density at 280 mu

Positive with Hassler's

Fig. 3 Reproduction by Skin Extract of Permeability Changes in Arthus Reaction

Course of Inflammation (hrs.)

● Skin extracts prepared at various intervals of Arthus reaction.
● Skin taken from rabbits with active Arthus reaction.

Extravascular Dye ( ug. )

Fig. 4 Demonstration of Permeability Factor in Skin Extract Eluted Through Sephadex Column
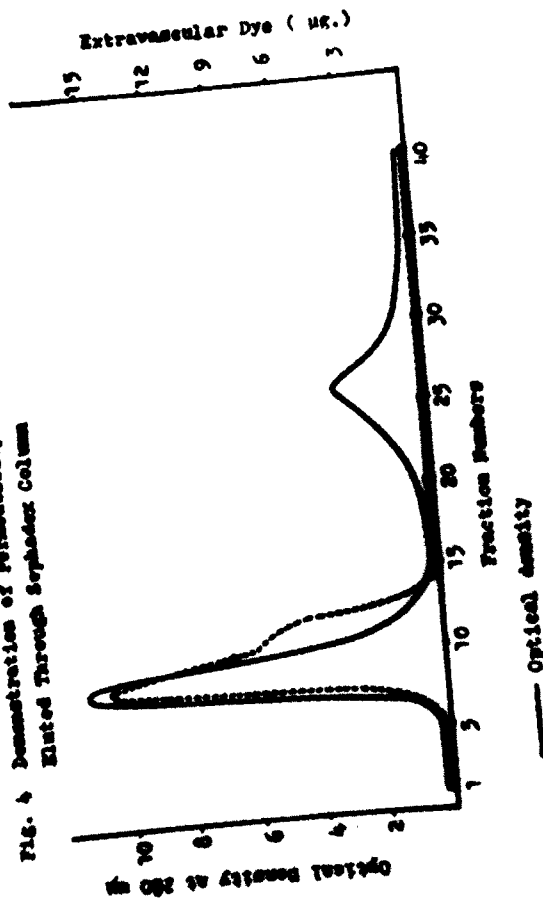
Fraction Numbers

Optical density

Optical Density at 280 mu

**Fig. 6  In vivo Effects of Heating, SBTI and DFP on the Inflammatory Permeability Factor of Pseudoglobulin**
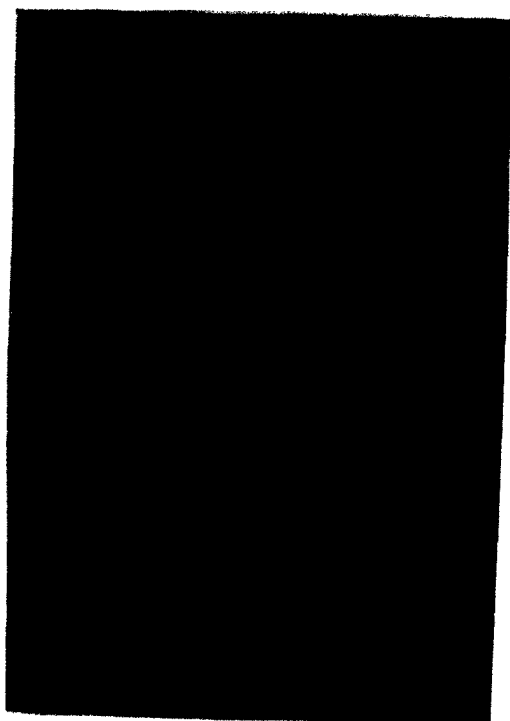


I-a, PF +  2.5 µg. SBTI
I-b, PF +  3.0 µg. SBTI
I-c, PF +  saline
II-a, PF +  10 M⁻⁴DFP
II-b, PF +  10 M⁻³DFP
II-c, PF +  saline
III-a, PF heated at 75°C, 30 min.
III-b, PF heated at 100°C, 5 min.
III-c, nonheated PF

Note no inhibitory effects of SBTI and DFP.  Complete loss of PF activity is shown by heating, 75°C, 30 min.; 100°C, 5 min.



Fig. 7  The skin site at 1½ hrs. after intradermal injection of inflammatory PF (0.1 ml.).  No extravascular migration of leucocytes.  However, there is seen considerably marked oedema.  H. and E. x 150

| | Anti-histamine | SBTI | DFP | Heating | Ileum contraction | Dialysis | Extravas. migration | Caseinolysis |
|---|---|---|---|---|---|---|---|---|
| Peptides: | | | | | | | | |
| Leukotaxine | - | | | - | - | + | + | |
| Bradykinin[2] | - | | | - | + | + | + | |
| Kallidin[3] | - | | | - | + | + | + | |
| Enzymes: | | | | | | | | |
| Globulin PF[4] | - | + | + | + | - | - | + | - |
| Kallikrein[5] | - | + | + | + | - | - | + | - |
| Our inflammatory PF: | - | - | - | + | - | - | - | - |

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TABLE II. Comparison of Permeability Effect of Inflammatory PF
Isolated at Various Intervals of Arthus Reactions

| PF samples | O.D. of samples* | Extravascular dye |
|---|---|---|
| 30 min. Arthus | 10.2 | 8.1 ug. |
| $4\frac{1}{2}$ hr. Arthus | 10.5 | 24.3 ug. |
| 12 hr. Arthus | 11.0 | 10.3 ug. |

* O.D., Optical density at 280 mu.

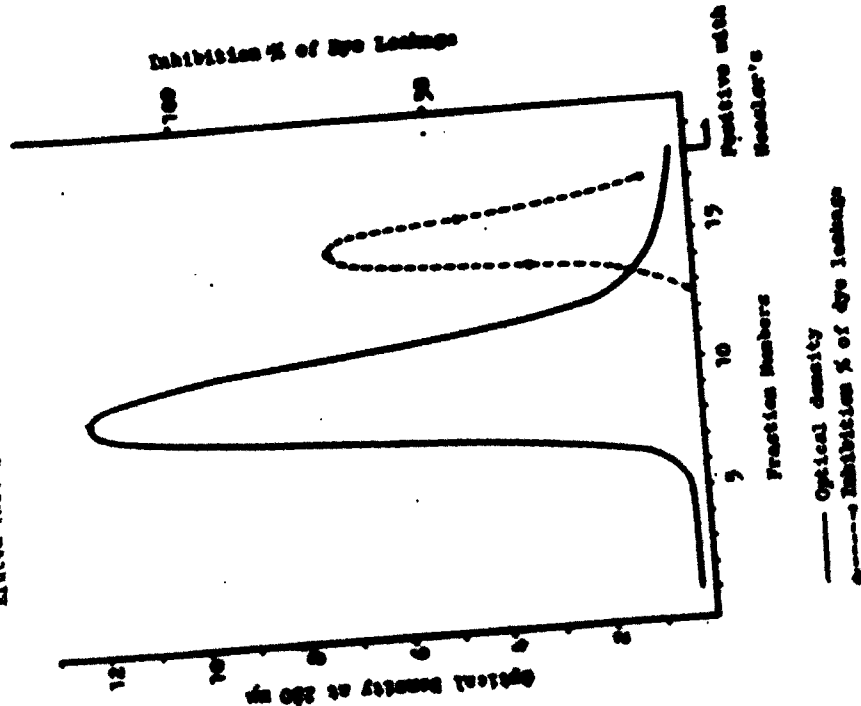Fig. 7 Demonstration of Permeability Factor in Thermal Pseudoglobulin Eluted Through Sephadex Column



Fig. 8 Demonstration of Anti-PF in Euglobulin Eluted Through Sephadex Column
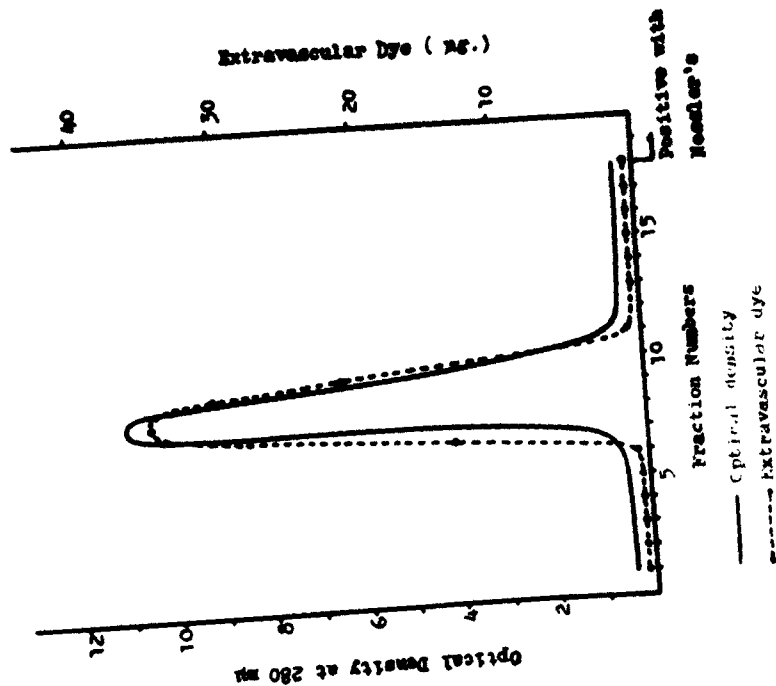
TABLE III. Effects of Our Anti-PF and Anti-protoase on Various
Permeability Factors

| Test materials | Concentration | Extravascular dye ( µg.) | Arthus Anti-PF Inhibition % | Thermal Anti-PF | Anti-protec |
|---|---|---|---|---|---|
| Leukotaxine | 2.5 mg./ml. | 12.6 | 0 | nt | nt |
| Bradykinin | 0.5 ug./ml. | 11.2 | 0 | nt | nt |
| Kallidin | 2 unit/ml. | 21.1 | 0 | nt | nt |
| Globulin PF | 1.5 mg./ml. | 14.4 | 0 | nt | nt |
| Kallikrein | 0.05 unit/ml. | 14.2 | 0 | nt | nt |
| Arthus PF | O.D. = 6.2 | 29.8 | 70 | 25 | 0 |
| Thermal PF | O.D. = 5.9 | 29.8 | 54 | 75 | 0 |
| Cellular PF* | 2-diluted. | 45.0 | 56 | nt | 0 |

* The PF was released into extracellular fluid (culture medium)
  by cultured fibroblasts submitted to antigen-antibody reaction.
  The fluid was taken 2 - 3 hrs. after introduction of antigen, it
  being diluted (Hayashi, H., 1962; Kinuwaki, Y. and Takaba, Y.,
  unpublished). Characteristic morphological changes in the
  cultured cells have been widely investigated (Hayashi, H. and Ono,
  T., 1955; Ono, T., 1958; Kuzo, T., 1960).
** Isolated from healing Arthus skin site and highly purified (see
  Udaka, K., 1960; Hayashi, H., 1962).